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THE GASES OF SWAMP RICE SOILS

PART III

A HYDROGEN-OXIDIZING BACTERIUM FROM THESE SOILS

BY

W. H. HARRISON, D.Sc

Government Agricultural Chemist, Madras

AND

P. A. SUBRAMANIA AIYER, B.A

Assistant to the Government Agricultural Chemist

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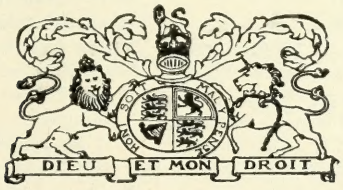

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THE GASES OF SWAMP RICE SOILS.

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PART I.

General.

In the recent Memoir published by Harrison and Subramania[1] dealing with the relationship between the gases of Swamp Rice Soils and the organized film present on the surface of the soil, it was shown that the latter possessed the power of oxidizing hydrogen. This oxidation was further demonstrated to be due to the activities of certain bacteria, and crude cultures were obtained and their action studied. In that Memoir it was intimated that a pure culture of a specific bacterium had been obtained, and it is with this organism that the present Memoir deals.

The crude cultures were cultivated and studied in Kaserer's solution[2] under autotropic conditions, and it was naturally assumed that the pure bacterium would also exist under these conditions and be similar in character, if not identical, with that isolated and described by Kaserer.[3] This, however,

[1] Harrison and Subramania. The Gases of Swamp Rice Soils, Part II, Their Utilization for the Aeration of the Roots of the Crop. *Mem., Dept. of Agri., India, Chem. Ser.*, vol. IV, no. 1.

[2] $K_2H\ PO_4$ ... 0·05%
$Mg\ SO_4$ .. 0·02%
Am Cl ... 0·10%
$NaHCO_3$ ... 0·05%
$Fe\ Cl_3$ ... trace.

[3] Kaserer. *Cent. Bakt.* 2 abt, 15 (1905) p. 573, 2 abt, 16 (1906) p. 481.

did not prove to be the case, for the organism which has been isolated from these rice soils develops only very poorly in pure culture under autotropic conditions and the oxidation of hydrogen only becomes prominent in the presence of small amounts of soluble organic matter.

On plating out the crude cultures on silica jelly, or washed agar media, containing the salts forming Kaserer's solution and incubating in an atmosphere consisting of a mixture of CO_2, CH_4, O and H exceedingly small colonies invariably developed which grew but slowly, but out of a large number specifically studied only one colony was found capable of oxidizing hydrogen under autotropic conditions and even so its action was slight :—

TABLE I.

Showing the oxidizing action of the colony under autotropic conditions.
cc. NT & P (Incubation period 9 days).

	Expt. I			Expt II		
	In the presence of CH_4			In the presence of CO_2		
	Before	After	Diff.	Before	After	Diff.
CO_2	.....	.....	.....	29·2	26·8	−2·4
CH_4	29·7	28·0	−1·7	.....	.....	.....
O	33·7	28·6	−5·1	33·1	28·8	−4·3
H	40·8	32·1	−8·7	40·0	31·6	−8·4
N	4·3	6·0	+1·7	4·2	4·9	+0·7

This culture grew fairly well on mineral agar medium, but microscopical examination revealed the fact that it was composed of a mixture of two different species of bacteria, one non-motile and the other smaller and a pseudomonas. All attempts to separate them by plating on mineral agar resulted in the production of colonies which had no action on hydrogen. The colonies thus isolated invariably contained the smaller organism present in the mixed culture and under these conditions it was not found possible to isolate the other bacterium.

A consideration of the above facts led to the conclusion that the hydrogen oxidation of the mixed culture was due either to two or more bacteria acting together in symbiosis, or that the particular bacterium to which the action could be ascribed was unable to exist in pure culture under autotropic conditions. In order to test the latter assumption the mixed culture was inoculated into Kaserer's solution to which 0·1 % of sodium asparaginate was added and the amount of hydrogen oxidation again determined. It was at

once evident that the addition of this organic matter had led to an increased amount of oxidation.

TABLE II.

Showing oxidation of H by mixed culture in 0·1 *Na. Asparaginate solution.* cc. NT & P (Incubation period 20 days).

	Before	After	Diff.
CO_2	*nil*	0·3	+ 0·3
O	23·6	*nil*	−23·6
H	63·1	15·7	−47·4
N	9·8	14·4	+ 4·6
Total	96·5	30·4	−66·1

This type of result confirmed the second supposition and the mixed culture was accordingly plated out on mineral agar, to which 0·1 % sodium asparaginate had been added, and incubated in an atmosphere of oxygen and hydrogen. Colonies developed of which a large number proved to possess the power of oxidizing hydrogen in the presence of soluble nitrogenous organic matter and which were found to consist of the larger non-motile bacterium previously referred to. These cultures were repeatedly re-plated in order to make perfectly certain that pure cultures were present, and the organism will hereafter be referred to as H_A. The motile bacterium associated with it in the mixed culture was designated as H_B.

The hydrogen oxidizing bacterium H_A thus isolated appears to be a new species. It is easily differentiated from Kaserer's organism, *B. pantotrophus*, as is shown in the following table :—

TABLE III.

Contrasting Bacterium H_A *with B. pantotrophus.*

	H_A	*B. pantotrophus*
Motility ...	absent	present
Flagellæ ...	absent	present
Colour on gelatine ..	white	yellow
Hydrogen oxidation ..	Practically absent under autotropic conditions; but pronounced in the presence of soluble organic matter.	Very pronounced under autotropic conditions but inhibited by the presence of soluble organic matter.

The characteristics of this bacterium are as follow :—

Morphological characteristics. Small bacterium, about 1-4μ long and 0·5μ broad which stains readily with ordinary aqueous stains, but the staining is however very uneven. Does not stain by Gram's method. It is non-motile and flagellæ and spores have not been demonstrated. In old cultures involution forms are common.

Gelatine stab (20% gelatine). A white, somewhat restricted surface growth is formed, which has a shiny wrinkled surface. Liquefaction has occasionally been noticed after several weeks. The growth along the line of the needle is poor and restricted to the portion near the surface of the gelatine.

Agar plates. White round colonies with entire edge and shiny wrinkled surface.

Potato. Moist shiny growth, very restricted and slightly raised at the edges. At first the colour is that of the medium but later it becomes a pale buff.

Broth cultures. Slight turbidity often with characteristic zoogloea masses floating in the liquid, later a tough pellicle develops. Indol is not produced.

Nitrate broth. Nitrates are not reduced and no gas is formed.

Glucose, Lactose, and Sucrose broth. No fermentation, neither acid nor gas being produced. Characteristic floating zoogloea masses are formed.

Milk is unchanged.

Chemical activities. The bacterium grows exceedingly poorly in mineral solution, or on solid mineral media under autotropic conditions in the presence of CO_2, $2CH_4$, H and O and at the same time only very small quantities of hydrogen disappear. But although practically inactive under these conditions yet in association with the bacterium H_B and water bacteria autotropic oxidation of H readily takes place. In this case, the bacteria are evidently existing together in a state of symbiosis, the foreign organisms assimilating the carbon-dioxide with the formation of organic matter which, in turn, enables the specific bacterium to carry out its functions.

Experiments under autotropic conditions were found to be unsatisfactory and discordant unless all traces of soluble organic matter were eliminated. It was found necessary to clean all apparatus with hot chromic acid mixture, to use only re-distilled water and to carefully re-crystallize all the salts used.

This bacterium grows quite well upon all ordinary organic nutrient media and under these conditions is able to oxidize hydrogen. This oxidation is evidently an energy source, but the intensity of the action is dependent not

only on the kind, but also upon the quantity of organic matter present. A large proportion of organic matter is particularly objectionable from this point of view as then the bacterium exists mainly upon the organic matter and the oxidation of hydrogen reaches a minimum.

The best results are obtained with solutions containing from 0·01 to 0·03% of such substances as peptone, nutrose, sodium asparaginate, etc. The organism is able to utilize ammonia and nitrates as the nitrogen source in presence of glucose, but in these circumstances little hydrogen oxidation takes place, and neither are nitrites nor nitrogen produced from the nitrate. It would almost appear as if the hydrogen oxidation went hand in hand with assimilation of organic nitrogenous food materials.

Association with H_B and water bacteria is just as effective in increasing the oxidizing action of the bacterium in organic media as was found to be the case under autotropic conditions. In fact, the only cases in which the reaction has been carried out to completion have occurred with such associations and the efficiency of these symbiotic relationships under natural conditions is thereby emphasized.

The main difficulty experienced during the investigation was due to the fact that the bacterium rapidly lost all oxidizing power when continuously cultivated on organic media. To obviate this, two methods were adopted, namely, (1) to grow the cultures on mineral agar to which not more than 0·03% sodium asparaginate was added and (2) to cultivate the mixed culture of H_A and H_B under autotropic conditions and to isolate the former in asparaginate agar as required.

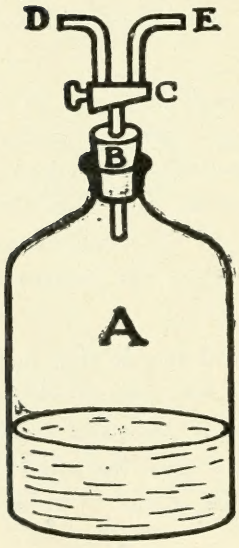
The functions of these oxidizing bacteria with regard to rice soils have been fully dealt with in the Memoir by Harrison and Subramania previously referred to and there is, therefore, no occasion to refer to it again here except to point out that they are the means of conserving the energy dissipated by the decay of organic matter in these soils, and making that energy, or at least part of it, available for the crop.

PART II.

Experimental.

Method of experiment. The experiments with the crude cultures were carried out with the apparatus and in the manner described in the Memoir previously quoted. But the impossibility of obtaining a sufficiency of similar apparatus to enable a large number of experiments to be carried out simultaneously compelled the adoption of a simpler method of experiment.

The apparatus used throughout the experiments dealt with in the sequel is shown in the annexed sketch.



The neck of a bottle A was closed with a rubber cork B through which a three-way glass stopcock C was inserted, the exit tubes D and E of which were plugged with sterile cotton wool. About 30 cc. of the nutrient solution experimented with was introduced into the bottle and the cork and stopcock then inserted and the whole sterilized in the autoclave.

The liquid was inoculated by momentarily removing the cork, and the exit tube D was then connected to an air pump and the exit tube E to a reservoir containing the mixed gases. The air was then pumped out of A until the liquid in it boiled. Gas was then admitted through E from the reservoir and the bottle was again evacuated. This was repeated 3 times in all, so as to make perfectly certain that the bottle was filled with the mixed gases.

After inoculating the bottles were stored under water and incubated at 34°C. At the end of this period the bottle was wiped dry and weighed and then the tap C was opened under water to allow the internal pressure to adjust itself. After bringing the surfaces of the internal liquid and external water to the same levels, the tap C was closed and the apparatus again wiped dry and weighed. Any difference in weight was equivalent to an alteration in the

volume of the gas in the bottle, which was reduced to NT & P both before and after the experiment. Finally, the gas was removed and analysed and any changes in composition which it had undergone noted. Knowing the composition and volume of the gas before and after the experiment it was possible to calculate the alteration in the volumes of the component gases.

Experiments under Autotropic Conditions.

(i). *H_A in pure culture.*

A very large number of experiments were carried out in which the bacterium was cultivated in Kaserer's solution in an atmosphere of CO_2, CH_4, O and H. As before indicated, in order to obtain concordant results it was found necessary to use redistilled water and carefully purified chemicals so as to obviate the presence of small traces of soluble organic matter which caused the bacterium to behave abnormally. It was also necessary to clean the apparatus used with chromic acid mixture.

The following table shows the type of result obtained :—

Table IV.

Showing the action of H_A in pure culture under autotropic conditions.

cc. NT & P.

Experiments	Expt. I			Expt. II			Expt. III		
Days incubation	10			20			10		
	Before	After	Diff.	Before	After	Diff.	Before	After	Diff.
CO_2	27·5	21·6	−5·9	2·0	1·7	−0·3	26·3	20·2	−6·1
O	57·5	51·3	−6·2	38·4	35·5	−2·9	32·1	27·1	−5·0
H	53·9	54·3	+0·4	48·9	50·5	+1·6	28·2	19·4	−8·8
CH_4	3·9	3·5	−0·4	42·6	39·4	−3·2	20·5	18·7	−1·8
N	4·2	4·1	−0·1	13·0	10·6	−2·4	27·8	25·6	−2·2
Total	147·0	134·8	−12·2	144·9	137·7	−7·2	134·9	111·0	−23·9

The bacterial growth in the bottles was exceedingly poor in all cases. From these results it is concluded that the bacterium possesses very slight powers of assimilating CO_2 and CH_4 under autotropic conditions, but that its power of oxidizing hydrogen is very small.

(ii). *H_A symbiosis with other organisms.*

The fact that the mixed culture of the bacteria H_A and H_B could, under autotropic conditions, oxidize hydrogen led to the study of their action under symbiotic conditions. In all cases, considerable oxidation occurred and much carbon dioxide disappeared.

TABLE V.

Showing the effect of H_A in symbiosis with H_B.

cc. NT & P.

	H_B alone 14 days			H_A + H_B 20 days		
	Before	After	Diff.	Before	After	Diff.
CO_2	49·6	30·5	-19·1	29·4	7·9	-21·5
O	75·2	85·2	+10·0	35·2	*nil*	-35·2
H	115·4	115·5	+ 0·1	103·0	7·5	-95·5
CH_4	32·4	27·7	- 4·7	17·9	16·5	- 1·4
N	14·3	17·0	+ 2·7	27·5	31·4	+ 3·9
Total	286·9	275·9	—11·0	213·0	63·3	-149·7

It would thus appear that the bacterium can exist under autotropic conditions in symbiosis with bacteria capable of assimilating either CO_2 or CH_4.

EXPERIMENTS UNDER HETEROTROPIC CONDITIONS.

The early experiments with the bacterium were carried out in solutions containing only very small amounts of organic matter varying from 0·01 to 0·05% but in no case, even with 30 days incubation, did the action proceed to completion, *i.e.*, when either all the H or O was used up. It was thought that by increasing the proportion of organic matter such would occur, but in reality it was found that the amount of hydrogen oxidized was greatly reduced. These facts led up to a study of the effect of varying proportions of organic matter upon the course of the reaction.

Cultures grown in Botkin Flasks with different kinds of organic matter gave different results. With substances of the type of peptone and nutrose a very good growth was obtained and the rate of oxidation was rapid. Sodium

asparaginate and asparagin gave inferior results, but still the action was marked. Glucose in conjunction with ammonium salts or nitrates yielded very inferior results and the hydrogen oxidation was very small. Oxidation of hydrogen by the bacterium appears to be only efficient when organic nitrogen is present although assimilation of N takes place from inorganic substances.

Solutions containing sodium asparaginate being entirely synthetic in character and the organic matter of a comparatively simple type experiments were first instituted with this substance to test the effect of different proportions. Later similar series were carried out with peptone.

(i) *The Effect of Varying Proportions of Sodium Asparaginate on the Oxidation of Hydrogen.*

With proportions of sodium asparaginate varying from 0·01 to 0·03% the oxidation of hydrogen proceeds smoothly and is not complicated by the presence of other actions. With stronger solutions oxidation of organic carbon with the production of carbon dioxide takes place in addition.

The following is the record of an experiment carried out in Kaserer's solution containing varying proportions of sodium asparaginate :—

Table VI.

Showing the action of the Bacterium in solution containing different proportions of sodium asparaginate.

No.	1			2			3			4			5		
% asp.	0·5			0·25			0·1			0·05			0·03		
	Before	After	Diff.	Before	After	Diff.	Before	After	Diff.	Before	After	Diff.	Before	After	Diff.
CO_2	1·4	26·9	+25·5	1·1	11·8	+10·7	1·1	10·3	+ 9·2	1·1	3·2	+ 2·1	3·6	4·4	+ 0·8
O	115·1	43·6	−71·5	95·4	63·6	−31·8	89·2	68·2	−21·0	94·2	81·4	−12·8	107·5	83·8	−23·7
H	151·0	119·0	−32·0	125·0	101·5	−23·5	117·0	98·2	−18·8	123·5	97·2	−26·3	152·3	100·5	−51·8
Total	267·5	189·5	−78·0	221·5	176·9	−44·6	207·3	176·7	−30·6	218·8	181·8	−37·0	263·4	188·7	−74·7

In the following table the important relationships of this experiment are brought forward :—

Strength of solution	0·5	0·25	0·1	0·05	0·03
Decrease in volume per 100 cc. of original gas ...	29·2	20·1	14·8	17·7	28·3
Production of CO_2 ,, ...	9·6	4·8	4·4	1·5	0·3
Disappearance of O ,, ...	26·8	14·3	10·1	5·8	9·0
Disappearance of H ,, ...	11·9	10·6	9·1	12·1	19·6
O/H Ratio .	1/·45	1/·74	1/·9	1/2·05	1/2·18

Thus the bacterium in dilute solution brings about only oxidation of hydrogen, whereas, with increasing strengths more and more carbon dioxide is produced and this is naturally followed by an increasing consumption of oxygen. The consumption of hydrogen, which can be taken to measure the amount of hydrogen oxidation, decreases at first with increasing amounts of organic matter and reaches a minimum with about 0·1%. Greater strengths than this bring about an increased hydrogen consumption but not in proportion to the oxygen consumption or the carbon-dioxide production. A similar phenomenon will be noticed when the effect of peptone is considered.

The best results so far as purely hydrogen oxidation is concerned are obtained with solution containing about 0·02% sodium asparaginate but in no case has the reaction been observed to go to completion. Complete removal of the hydrogen or oxygen is, however, attained with mixed cultures with water bacteria. The following results were obtained when a few drops of the water used for irrigating the rice fields were added to the experiment bottles :—

TABLE VII.

Showing action of the Bacterium in association with Water Bacteria.

cc. NT & P.

	H_A and water bacteria			H_A alone		
	Before	After	Diff.	Before	After	Diff.
CO_2	1·5	0·4	− 1·1	1·6	2·8	+ 1·2
O	45·5	13·5	−32·0	48·3	39·0	− 9·3
H	64·8	*nil*	−64·8	67·2	51·1	− 6·1
N	1·0	5·1	+ 4·1	1·0	8·3	+ 7·3
Total ...	112·8	19·0	−93·8	118·1	101·2	−16·9

Consequently, as under autotropic conditions, the bacterium reaches its highest efficiency when living in symbiosis with other organisms.

(ii) *The Effect of varying Proportions of Peptone on the Oxidation of Hydrogen.*

Several series of experiments with varying amounts of peptone were carried out in a similar manner to those with sodium asparaginate. The record of one such series is given below ;—

TABLE VIII.

Showing the effect of varying proportions of peptone on the oxidation of hydrogen. cc. NT & P.

% Peptone	0·5			0·25			0·125			0·062			0·031		
	Before	After	Diff.	Before	After	Diff.	Before	After	Diff	Before	After	Diff.	Before	After	Diff.
CO_2	1·0	0·8	- 0·2	1·0	4·1	+ 3·1	1·0	9·8	+ ×·8	0·9	2·8	+ 1·9	1·0	1·9	+ 0·9
O	55·7	45·1	-10·6	55·3	40·8	-14·5	53·4	16·4	-37·0	51·5	11·5	-40·0	55·3	31·3	24·0
H	84·5	60·5	-24·0	84·1	68·3	15·×	81·0	48·1	-32·9	7×·3	13·6	64·7	84·0	40·5	-43·5
N	2·8	3·1	+ 0·3	2·7	4·7	+ 2·0	2·6	4·8	+ 2·2	2·5	3·7	+ 1·2	2·7	3·8	+ 1·1
Total ...	144·0	109·5	-34·5	143·1	117·9	-25·2	138·0	79·1	-58·9	133·2	31·6	-101·6	143·0	77·5	-65·5

These results when collated in a similar way to the ones with sodium asparaginate yield the following comparison:—

	0·5	0·25	0·125	0·062	0·031
Disappearance of gas per 100 cc. of original volume	24·0	17·6	41·7	87·0	45·8
Production of CO_2 ,, Do.	−0·1	2·0	6·4	1·4	0·7
Disappearance of O ,, Do.	7·4	10·1	26·8	30·0	16·9
Disappearance of H ,, Do.	16·6	11·0	23·9	48·6	30·8
$\frac{H}{O}$ ratio	2·2 / 1	1·1 / 1	0·9 / 1	1·6 / 1	1·9 / 1

These results show a very similar variation in the effect of the organic matter on the hydrogen oxidation to those with sodium asparaginate. The main difference being that from peptone there appears to be comparatively little CO_2 production but this fact, however, makes clearer the relative intensity of the hydrogen oxidation.

(iii) *The Action of the Bacterium on Hydrogen with Ammonia and Nitrates or the Nitrogen source.*

The results previously considered have been those in which organic nitrogen supplied the necessary nitrogen, but the ones now considered deal with the action when the nitrogen is derived from purely mineral sources.

The first results given are those in which ammonia alone is the nitrogen source and glucose providing the carbon source.

TABLE IX.

Showing action of Bacterium H_A with ammonia as the nitrogen source and glucose as the carbon source.

cc. NT & P.

	Before	After	Diff.
CO_2 ..	0·3	11·0	+10·7
O ..	69·2	48·3	−20·9
H ..	84·0	72·3	−11·7
N ...	3·7	2·7	− 1·0

The organism can evidently assimilate ammonical N in the presence of glucose and a fair amount of hydrogen is oxidized. There is also a considerable production of CO_2 from the glucose.

TABLE X.

Showing action of Bacterium with nitric nitrogen as the nitrogen and glucose as the carbon source.

	Before	After	Diff.
CO_2 ..	0·3	7·2	+6·9
O ..	60·6	55·8	−4·8
H ..	73·8	72·1	−1·7
N ...	3·2	4·2	+1·0

A fairly good growth occurred, but the results clearly show that under these conditions little hydrogen is oxidized. At the same time no nitrous acid was produced.

COIMBATORE,
September 15, 1915.

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